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9.1

The development of mutations of tobacco mosaic virus by the chemical treatment of its nucleic acid in vitro.

by K. W. Mundry and A. Gierer.

Zeitschrift f. Vererbungslehre, 89: 614-630 (1958).

Results.

a) Preliminary tests. For the purpose of qualitative orientation, the virus which had been inactivated by several decimal powers by prolonged subjection to the effect of nitrite was tested on "Java" and "Xanthi" tobacco in a relatively high concentration. This is compared with diluted, non-treated virus. Table 1 shows that virus treated with nitrite in this manner causes a very great number of necrotic lesions on Java tobacco, while non-treated virus of the same infectivity (established on Xanthi plants) develops only a few necroses on Java plants. Fig. 1 b depicts a leaf of Java tobacco inoculated with vulgare TMV treated for only 20 minutes with nitrite. In addition to the chlorotic foci of infection by vulgare, several necrotic foci are distinctly visible.

It shall be shown in the following that these necrotic lesions must be attributed to mutants which were generated by the chemical treatment of the virus or of RNA in vitro.

b) Quantitative analysis. For the purpose of quantitative analysis, the time of subjection to nitrite was varied and the remaining conditions remained constant in the course of a time series. Table 2 contains such a metric series for isolated RNA of TMV strain vulgare. It is evident that the number of local necrotic lesions generated on Java tobacco at a constant RNA concentration initially experiences a sharp rise and then shows in the further course of incubation. The fraction of necrotic lesions among the total number of all foci of infection continues to rise steadily.

According to tests by Schuster and Schramm, inactivation with nitrite occurs by the chemical alteration of individual nucleotides and, therefore, exponentially with time:

$$n = n_0 e - \frac{t}{\tau} \qquad (1)$$

(n= concentration of infectious particles after time t; n_0 = concentration of infectious particles at time t = 0).

Time τ defines that time after which the number of infectious particles has decreased to 1/e, i.e. to 37% of the original value. On the basis of the series of dilutions, the corresponding concentration n of infectious particles may be determined for each value of infectivity

following incubation with nitrite. Time τ is then obtained according to equation (1) by applying the log of the concentration against the time. For example, the inactivation curve in Fig. 3 is thus obtained on the basis of the metric values of table 2. It is evident that the values measured on Xanthi and Java tobacco yield a common curve and support each other in this manner. For the test tabulated in table 2 and Figs. 2 and 3, a τ of 18 minutes is obtained.

If the induction of mutants is due to chemical alteration of single nucleotides, as in the case of inactivation, and p mutagenic changes of the searched-for phenotype (phaen) are obtained for each inactivation, a linear increase in the fraction of mutants among the "surviving" virus particles may be expected, according to the equation

$$\frac{m}{n} = p \cdot \frac{t}{\tau} \qquad (2)$$

(m = concentration of necrotizing mutants).

The total concentration of mutants, on the other hand, is obtained by combining of equation (1) with equation (2) to form

$$m = p \frac{t}{\tau} n_0 e^{-\frac{t}{\tau}} \qquad (3)$$

or, applied to the maximal value of m

$$\frac{m}{m_{max}} = e \cdot \frac{t}{\tau} \cdot e^{-\frac{t}{\tau}} . \qquad (4)$$

It is likely that equations (2) and (3) are valid only for a limited period of time t, since saturation effects are possible after prolonged treatment with nitrite. Equation (3) will represent the approximate dependence on the time factor of the necrotic lesions developing due to the effect of nitrite, as long as the number of necroses is nearly proportional to the concentration of mutants.

However, if the mutations were due to the simultaneous alteration of several (namely ν) nucleotides, then a relation of the following type should result:

$$\frac{m}{n} = \text{const.}\ t^{\nu} , \qquad (5)$$

$$m = \text{const.}\ t^{\nu} \cdot e^{-\frac{t}{\tau}} . \qquad (6)$$

The preceding equations make it possible to describe measurements conducted under dissimilar conditions in a uniform manner. This is done by reducing all points of measure to the time τ which corresponds to the conditions, time τ being measured independently. The same fraction of chemically altered bases in RNA corresponds to the same value $\frac{t}{\tau}$.

In this manner, measurements taken under different conditions may be compared. First, as depicted by the preceding example, time τ has been established for every individual series of measurements on the basis of the inactivation curve. Then the number of visible necrotic lesions --- applied to the corresponding maximal value --- was determined for each experiment, separately and in relation to time. In Fig. 4 the values thus obtained are applied against t/τ and compared with the theoretical curve, equation (4), of the one-hit reaction. There is favorable agreement. This applies both to the measurements of vulgare TMV and the RNA isolated therefrom under two different conditions, and to the RNA of strain B11 (*). The decision in favor of the one-hit reaction is supported especially by the characteristic starting values in connection with short incubation times. The linear rise of the experimental values strongly deviates here from the parabolic rise of the multi-hit curve (Fig. 4). In Fig. 5 the fraction of the necrotizing foci of infection among the total number of all foci on Java tobacco is entered in relation to time, where the reduced time τ is again chosen as the unit of time. Here, too, the initially linear rise of induced necrotic lesions with time, corresponding to the one-hit reaction, is evident. The subsequent bend in the curve may possibly be ascribed to the indicated effect of saturation. Strain B11 of TMV also shows the linear rise of the necrotic portion with time. For the characteristic time $t = \tau$, an approximate ratio of 6% mutants is found among the "survivors" in the case of vulgare, and about 2% for B11. In the event the "plating efficiency" is not changed by the mutation, this corresponds to the relation p of the mutagenic hits to the lethal hits, according to equation (2). Since, according to Schuster and Schramm, the alteration of every single one of about 3,000 nucleotides has a lethal effect, there would be 6% in the case of RNA of strain vulgare, i.e. 180 nucleotides whose change would lead to a mutant with the characteristic of Java necrosis. The antecedent of this evaluation still requires experimental confirmation, however.

(*) One of the numerous strains developed by spontaneous mutation from vulgare isolated in the meanwhile by Melchers, characterized by primary chlorotic lesions and light yellow secondary symptoms with small necroses.

The congruent course of the corresponding curves for RNA and intact virus already indicate that the albumen fraction of the virus has no decisive influence on the reaction's mechanism.

In order to confirm this, the rate of spontaneous mutation to necrotizing mutants on Java tobacco was first determined both for intact vulgare TMV and infectious RNA prepared from it. The two rates obtained

are identical (table 3). The same test was conducted in another experiment with nitrite-treated vulgare whose infectivity had been reduced to about 1/e of the original value (t approximately = τ). Again, protein was removed from a part of this nitrite-treated virus by fourfold shaking with phenol; the RNA obtained was tested as above. In the case of this nitrite-treated virus, too, no differences between the mutation rates of intact virus and the RNA subsequently prepared from it were noted. The two rates are identical to each other, but considerably greater than the spontaneous rate of untreated virus (table 3). This shows that the observed effect is due to the influence of nitrite on the RNA of the virus, and that its quantitative result is independent of the presence of virus protein.

c) The biological proof of the mutagenic effect of nitrite incubation. As the quantitative analysis indicates, the genesis of necrotic lesions on Java leaves induced with nitrite follows laws that suggest a direct chemical, i.e. mutagenic, alteration of RNA via single nucleotides. In addition, it was necessary to prove the development of new strains as evidence of a genuinely mutagenic effect. In this connection, the stability of newly appearing characteristics, in particular, had to be tested in transplanting experiments. Then we had to examine whether, in addition to the primary ability to necrotize Java tobacco, other new characteristics appear in the wake of treatment with nitrite.

These two problems were investigated in the course of an extensive inoculative experiment.

The principle of this experiment is the isolation of individual foci under conditions that preclude the possibility of subjective selection, and the transfer of virus from these foci to plants whose symptoms, following pathogenesis, permit the observation of as many viral characteristics as possible. We proceeded with the groups "RNA incubated with nitrite for 96 minutes" and dilution control with 0.19 γ RNA/ml of the test summarized in table 2. The infectivity (according to the number of necrotic lesions per Xanthi leaf) of both is nearly equal and the number of necrotic lesions per leaf (about 3) so low that foci of infection may be isolated with ease. They were cut out and homogenized in tissue micro-mortars. The homogenate of each individual necrotic lesion was diluted to 1 ml with buffer and frozen. On the following day the melted homogenates were used to inoculate one Samsun and one Java seedling each per original Xanthi necrotic lesion, using a glass spatula and carborundum. In this manner all Xanthi necroses of the two comparative groups were isolated, totalling 60 in the "96 minute nitrite group" (labeled with numbers 1-60) and 65 in the diluted controls with 0.19 γ RNA/ml (numbers 61-101, 101a, 102-124).

The results of this test are summarized in table 4 and Figs. 6 and 7. In contrast to the uniformly growing controls, the result in the case of nitrite-incubated RNA is entirely different qualitatively as well as quantitatively: In this group, 20 of 60 single focus transfers have failed, in the controls only 1 among 65; of the 40 infections from necrotic lesions of the nitrite group, only 7 cannot be differentiated

from the controls by their symptoms.

These results remained unchanged after a second transplantation which was conducted 2½-3 weeks later from diseased plants of Samsun and Java tobacco onto young samsun seedlings. The symptoms noted after this second transfer were identical to those of the Samsun plants of the first transplant. Samsun seedlings inoculated with virus material from Java plants devoloped the same symptoms as those of the corresponding parallel inoculation from Samsun plants. This proves that the differentiating symptoms must be ascribed not to physiologically caused variability of the host plants, but to genetically fixed, new characteristics of the virus. --- The mutation rate for the necrotizing character amounts to 15% (cf. table 2) under the conditions of this incubation. The general incidence of mutants proves to be much higher upon evaluation of a maximum number of phonotypes (phaene), as was done in this experiment. It is possible that a more precise examination may prove that RNA incubated with nitrite for 96 minutes under these conditions no longer contains any material that still maintains the unaltered genetic character of the starting material.

TABLES.

Table 1. Effect of incubation of vulgare TMV not necrotizing Java tobacco on the necrotizing ability of this virus.

Concentration in the incubate: 4.4 mg virus/ml; pH during incubation: 4.1; nitrite concentration in the incubate = 1 molar; dilution after 1, 2, 3 and 4 hours with m/15 phosphate buffer pH 7.0 to 0.44 mg virus/ml, then dialysed against the same buffer and inoculated on Java tobacco leaves.

Group		TMV concentration in the inoculum (10^{-6} g/ml)	1st test necroses/leaf on Xanthi	1st test necroses/leaf on Java	2d test necroses/leaf on Xanthi	2d test necroses/leaf on Java
Incubated with $NaNO_2$ at pH 4.1	1 hr	440	558	345	277	113
	2 hrs	440	187	53	37.5	25.2
	3 hrs	440	2	1	8.21	8.4
	4 hrs	440	0.36	0.29	-	-
non-treated dilution control		4.4	409	1.9	257	1.4
		0.44	163	0.5	56	0.63
		0.044	26.3	0.16	8.6	0.13
		0.0044	2.08	0.045	0.8	not tested

Table 2. Dependence on the duration of subjection to nitrite of the genesis of local necrotizing infective foci from non-necrotizing vulgare RNA.

Concentration in the incubate: 9.5×10^{-4} g RNA/ml; pH in the incubate: 4.3; nitrite concentration in the incubate = 1 molar. Dilution after 1-96 minutes with M/15 phosphate buffer pH 7.0 to 1.9×10^{-5} g RNA/ml.

RNA concentration (10^{-6} g/ml)	Duration of incubation (min)	Xanthi necroses per leaf	Java necrotic & chlorotic lesions per leaf= total P.S.	Java necroses per leaf	Java necroses X 100 / total P.S. (%)
19	1	72.8***	183***	1.4	0.77
19	4	72.4***	130***	2.5	1.9
19	8	89.8***	180***	4.5	2.4
19	16	59.3	97	5.4	5.6
19	32	36.1	63	6.6	10.5
19	64	10.4	21.4	2.1	9.8
19	96*	2.72	3.5	0.54	15.5
19	0	1.5	137.5	0.29	0.21
1.9	0	1.2	41.7	0.13	0.31
0.19**	0	3.06	10.4	0	0
0.019	0	0.28	0.75	0	0

* In this group a total of 60 Xanthi necroses were counted. They yielded the starting material for the inoculative experiment described in table 4. The mutants depicted in Fig. 6 originated in this group.

** In this group a total of 65 Xanthi necrotic lesions were counted. They yielded the starting material for the controls of the inoculative experiment of table 4 and the controls depicted in part in Fig. 7.

*** For the determination of t' (i.e. the inactivation curve), the mean was drawn from these values and those of the first control (19×10^{-6} g RNA/ml) and the mean was utilized as the 3 minute value.

Table 3. Comparison of spontaneous appearance of local necrotic lesions on Java tobacco with that elevated by the nitrite incubation of intact vulgare TMV, for unimpaired TMV and its isolated RNA.

Test #	Preparation	Concentration of TMV or RNA in 10^{-6} g/ml	Java necroses & chloroses per leaf	Necrotic lesions per leaf	Mutation rate %
1	TMV vulgare, non-treated	0.26	80	0.15	0.187
	RNA from non-treated vulgare TMV vulgare, incubated 20 min with nitrite	7.0	111	0.2	0.180
		0.7	81.3	4.6	5.65
2	TMV vulgare, incubated 20 min with nitrite	0.7	40.7	1.35	3.32
	RNA from vulgare TMV incubated 20 min with nitrite	20.0	54	2.12	3.93

Table 4. Summary of the types deviating from non-treated starting material found in vulgare RNA incubated in nitrite after testing on Samsun and Java tobacco (test data see table 3).

Designation of symptom types	Preparation = RNA from vulgare TMV	Cf. Fig. 6	Treated w/nitrite 96 min		Non-treated dilution control	
	Test plants		20×10^{-6} g RNA/ml Samsun*	Java*	0.19×10^{-6} g RNA/ml Samsun**	Java
	Inoculation failed	-	20	17	1	1
	Probably unaltered virus	-	7	12	64	64
	Similar to vulgare, but without significant deformation of leaves	c	5	2	0	0
	Types with distinct striated venation	d	7	5	0	0
	Marked strains	g	2	0	0	0
	Diffusely spreading mosaic types	-	3	6	0	0
	Attenuated types	h	2	2	0	0
	Strains with strong (to severe) deformation of leaves **	e	3	2	0	0
	Light green mosaic strains	-	4	1	0	0
	Systemically necrotizing strains	a	2	0	0	0
	Atypical single forms	b,f	3	1	0	0
	With primary infections only: -					
	a) necrotizing		1	4	0	0
	b) chlorotizing		1	2	0	0
	c) weakly chlorotizing		0	5	0	0
	d) [illegible] if prim.infection		0	1	0	0
	Total		60	60	65	65
	Single focus inoculations done		60	60	65	65

p.7.

Footnotes to table 4:

* A symptomtype found on Samsun tobacco does not necessarily produce the same symptoms on Java tobacco. This is true especially of weak strains.

** Reduction of lamina in part up to center rib. Such severe injuries become fully evident only 2-3 weeks after infection.

*** Cf. Fig. 7.

ILLUSTRATIONS.

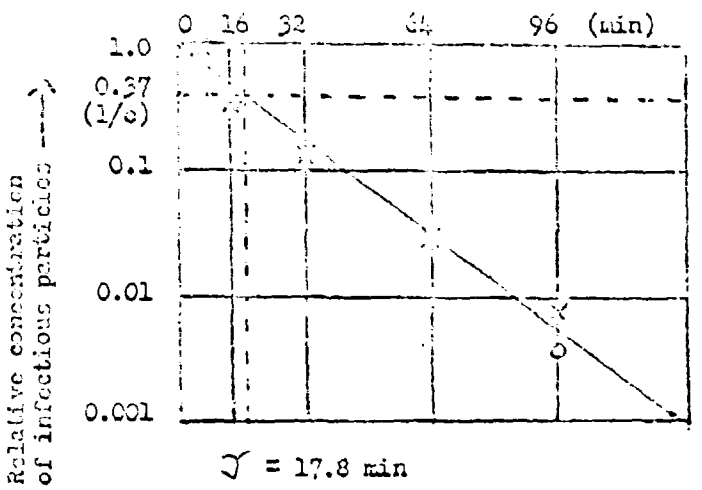

Fig. 3. Inactivation curve to data from table 2; for tests with Java tobacco o-------o, on Xanthi tobacco x-------x. The time required for inactivation to 37% yields the "reduced time τ ".

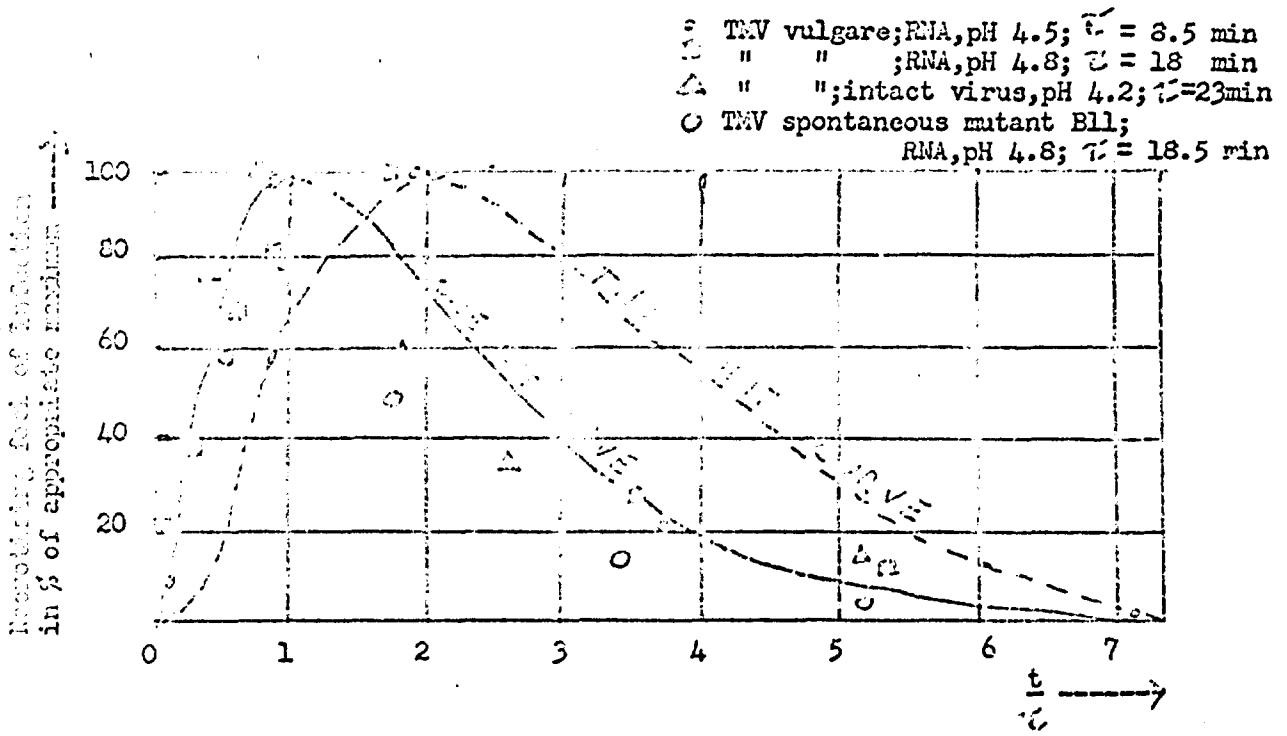

Fig. 4. Relation between the absolute number of necrotizing mutants noted per leaf and the duration of the influence of nitrite, the latter represented by the quotient "duration of treatment: reduced time τ " (cf. Fig. 3 for determination of τ).

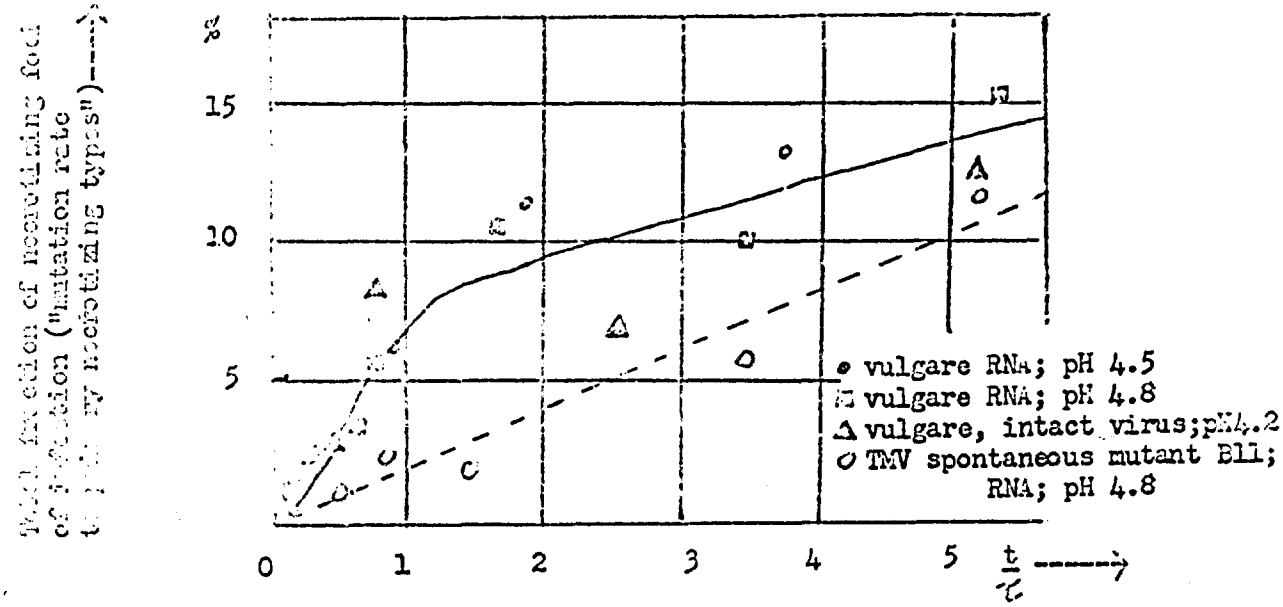

Fig. 5. Relation of the fraction of necrotizing types among the total of all infective foci on Java tobacco (necrotic + chlorotic lesions, cf. Fig. 1 b, = "mutation rate") to the duration of treatment with nitrite in connection with 2 strains of TMV. Sections of abscissa same as in Fig. 4.

Fig. 6 a-h. Some examples of TMV strains developing after incubation of TMV vulgare RNA with nitrite and isolated from necrotic lesions of Xanthi tobacco leaves. Summary of mutants obtained in table 4; experimental data in table 2 and text pp. 623 and 626. --- a) systemically necrotizing on Samsun, Ni 45; b) atypical single form Ni 53; c) similar to vulgare, but without distinct deformation of leaf Ni 20; d) striated venation type Ni 18; e) with increased deformation, in later stadia reduction of the lamina as far as the center rib, Ni 10; f) atypical single form Ni 14; g) masked strain Ni 54; h) attenuated strain Ni 19.

Fig 7 a-h. Selected examples of extreme variability within a control group with non-treated RNA. Inoculations from necrotic lesions of Xanthi tobacco leaves. Summary and comparison with nitrite-treated RNA see table 4 and Fig. 6, experimental data table 2 and text pp. 623 and 626. --- a) control 63, b) control 101, c) control 109, d) control 75, e) control 111, f) control 85, g) control 71, h) control 61.

Discussion.

In order to demonstrate the genesis of mutants in vitro, conditions were chosen under which a strong increase occurs in the number of necrotic lesions caused by the mutants on Java tobacco. Owing to this increase, the number of mutants multiplies 20 times over the original value in an area where the infectivity has decreased by only one half. This precludes selective processes. Nor may the rise be attributed to a higher degree of probability of the mutants' assertiveness due to dilution or inactivation, as frequently noted (Kundry 1957 c). Therefore the mutants were induced by treatment with nitrite.

In the case of equal representation of altered bases, i.e. after identical inactivation, e.g. to 1/e, the percentage of mutants among the infectious particles is the same for isolated RNA treated with nitrite, for virus treated with nitrite, and for RNA subsequently isolated from it. Thus the mutagenic effect does not affect the protein and has no connection with aggregational phenomena, which are entirely different for RNA and TMV; therefore chemical changes of RNA must be involved here.

Quantitative analysis indicates that the change of a single nucleotide in the chain of 6,000 suffices to evoke a mutation. Numerous, probably hundreds of nucleotides lead to mutants upon alteration. Genetic examinations indeed showed that a multitude of different, genetically anchored phenotypes (phaene) appeared as a result of treatment with nitrite.

While the tests positively indicate that the chemical alteration of RNA in vitro leads to mutants, it was found also that the removal of protein from the virus is not followed by a change in the mutational behavior. The spontaneous rate of mutation in the direction of the necrotizing character on Java tobacco is approximately the same for TMV and RNA (see table 3), namely, 0.2% under our conditions. Inoculations of 65 single foci caused by infectious RNA on Xanthi tobacco also failed to reveal altered symptoms, in contrast to RNA treated with nitrite.

As already mentioned, the mutagenic effect of nitrite is independent of oxygen. These findings [illegible] quantitative data in the literature, according to which [illegible] are known to be more frequent upon infection with [illegible]. It is [illegible] probability that RNA, by combining with the protein of [illegible], leads directly to a genetically changed virus (reactivation of [illegible]).

The specific effect of nitrite lies in a chemical transformation of individual bases of the unimpaired chain of RNA. In the case of the change from cytosine to uracil, a base is changed into another base which also occurs naturally in RNA. In this case it is possible (though not proved) that the RNA chemically altered in the test tube, may multiply identically in virus propagation. Upon the reaction of adenine and guanine, hypoxanthine and xanthine are formed, which are not identical to any of the naturally occurring purines. Here the possibility is perhaps given that in virus reproduction, instead of the altered bases, an analogous, physiologically present base is substituted, e.g. guanine in place of the hypoxanthine resulting from adenine. A decision in favor of this possibility or other, more complicated relations between the changed RNA and their reproductive products is not yet possible. Independently of such specific mechanisms, our investigations reveal that the exchange of an NH$_2$-group for an OH-group on a single base of the RNA chain in vitro activates the production of genetically altered virus in the host cell.

These findings presumably facilitate the approach to some interesting problems. These include among others the mechanism of RNA production, its connection with albumen synthesis and the correlation between the RNA and protein components of virus. On the other hand they make available an easy method for the induction and selection of certain mutants, e.g. of attenuated virus strains. They also open the perspective on an accumulation of several steps of mutation to "attenuated," which, when applied to viruses pathogenic for man, such as poliomyelitis, means that active immunization with viable virus against "regressive mutation" could be secured.

[illegible]

several of the primary infections [illegible] (Fig. 1b arrows). In studying this in more detail the following results were found:

a) TMV strain *vulgare* is strongly inactivated by nitrous acid and produces many necrotic lesions (Table 1, Java tobacco).

b) If all conditions of the treatment except its duration were kept constant an increase of time of treatment leads to an absolute increase in the number of necrotic lesions per leaf exceeding the spontaneous rate at least 20 times, while the infectivity of the sample was reduced to only ∼ 50% (Table 2). This effect cannot be due to selection of pre-existing mutants but must be attributed to the production of mutants in vitro.

c) This production of mutants is independent from the presence of the protein component of the virus (Table 3, Fig. 4); the rate of mutation (= ratio of number of necrotic lesions on Java tobacco leaves to the number of necrotic *and* chlorotic lesions on these plants) is the same with intact virus treated with nitrite, isolated RNA treated with nitrite, and RNA isolated from nitrite-treated complete virus.

d) The quantitative relation between the production of mutants by nitrous acid and the duration of treatment of virus or RNA is that of an one-hit reaction (Fig. 4: [illegible] theoretical one-hit curve; - - - theoretical double-hit curve). Thus the necrotic character [illegible] appear when only one out of the 6000 nucleotides of the TMV-RNA is altered.

e) So far it can be judged by the symptoms developing on Samsun (= Turkish) tobacco and Java tobacco plants that only a little amount of RNA remains unchanged after treatment in 1 n $NaNO_2$ solution at p_H 4.8 for [illegible] hours. Not only the Java-necrotic type but *many* new characters arise (Table 4). Out of 60 local-lesion transfers from Xanthi tobacco, 40 produced infections on Samsun and Java tobacco plants, 20 were lost. Among 65 control transfers of single lesions from Xanthi tobacco only one fails to give an infection. The symptoms of only 7 of the 40 infections obtained from treated RNA seem to be identical with those of the controls. A selection of several virus strains found with nitrite-incubated RNA is shown in Fig. 6. Fig. 7 gives an impression of the uniformity of the controls although the types shown in this figure are selected for greatest differences in their symptoms.

All these experiments show that replacement of one single NH_2-group by one OH-group in vitro can change the genetic character of the whole TMV-RNA molecule.

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